(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 337 327 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 89106213.5

(51) Int. Cl.4: C08B 37/10 , A61K 31/725

(22) Date of filing: 07.04.89

(30) Priority: 09.04.88 ES 8801088
30.03.89 ES 8901109

(43) Date of publication of application:
18.10.89 Bulletin 89/42

(84) Designated Contracting States:
CH DE FR GB IT LI LU

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(54) Process for the preparation of new oligosaccharide fractions by controlled chemical depolimerization of heparin.

(57) The process for the preparation of new oligosaccharide fractions is characterized by the fact that the starting aqueous heparin solution is ultrafiltered prior to the depolimerization treatment in order to exclude from it fractions of molecular weight lower than 3000 Daltons. Then, a partition is performed between liquid phases in order to segregate fractions of low anionicity from the depolimerization treatment. All the steps are carried-out in liquid phase without intermediate isolates and the controlled chemical depolimerization can be performed in more than one cycle.

Applied to pharmaceutical products of antithrombotic activity with low haemorrhagic risk.

EP 0 337 327 A1

PRODUCTION OF OLIGOSACCHARIDE FRACTIONS HAVING ANTITHROMBOTIC PROPERTIES BY CONTROLLED CHEMICAL DEPOLYMERIZATION OF HEPARIN

This invention refers to a physico-chemical process of industrial preparation of an oligosaccharide fraction derived from commercial heparin and containing useful pharmaceutical properties, such as : high inhibition of the Xa factor, high antithrombotic, and low anticoagulant activities.

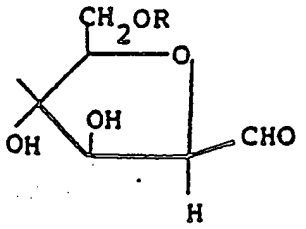
Heparin is a polydisperse polymer of the glycosaminoglycan family. Commercial products obtained from different sources (porkand beef mucose and bovine lungs) contain a mixture of polysaccharides with a continuous distribution of molecular weight in the range of 1500 to 30000 Daltons. Nader, H.B. et al. Biochem Biophys. Res. Commun. 57, 448-493 (1974).

Heparin produces a high anticoagulant action increasing the inhibitory effect of antithrombine III (AT III) in various activated factors of the coagulation cascade. This action is influenced by its molecular weight profile. It is verified that decreasing the molecular weight causes an increase in the ratio between the anti-Xa and antithrombotic activity.

It is known (Andersson L. et al Trombos. Res. 9, 575-583, (1976) Barrowcliffe T.W. et al. Thrombos. Res. 12 27-36 (1978), Lane D.A. et al. Trhombos Res. 12 257-271, (1978), that by fractioning the commmercial heparin of distinct origins in gelS such as Sephadex or Ultrogel Ac 44, different mean molecular weight fractions are obtained of a determined range which demonstrates an alteration in the anticoagulant activity according to methods such as USP XXI, BP or APTT and which are relatively more active against the Xa factor than the thrombine (Bjork etc.)

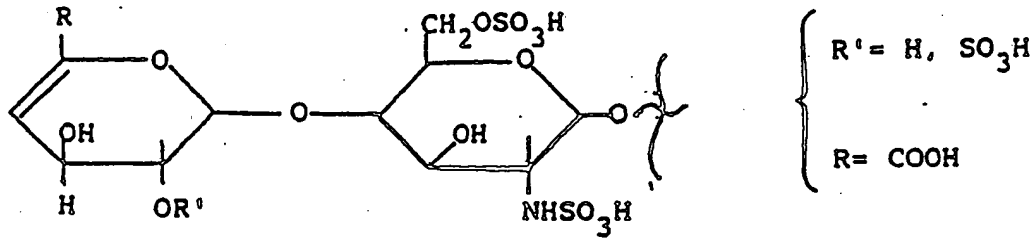
The essential pentasaccharidic sequence corresponding to the active site of binding between the heparin and the antithrombine is also well-known. The molecular weight fractions lower than 5000 Daltons (16-18 monosaccharides) which still contain this sequence are not long enough for simultaneous binding with the antithrombine and thrombine and can therefore, only neutralize the Xa factor.

Heparin's haemorrhagic risk which is more related to the platelet aggregation than to coagulation, is reduced with the low molecular weight fractions of heparin. (Westwick J. et al. Thrombos Res. 42, 435-447 (1986), Salzman, E.W. et al. j. Clin. Invest 65 64-73 (1980) have proved that low molecular weight fractions with high affinity for the ATIII (Antithrombine III) interact with platelets less than high molecular weight heparin fractions with high affinity to ATIII. Cade, N.F. et al. Thrombos Res. 35, 613-625 (1984) have demonstrated the lower inhibition effect of the low molecular weight heparins on the platelets' functions. Studies of Ostergaard, P.B. et al. Thrombos Res. 45, 739 (1987) have demonstrated that the doses of low molecular weight heparin necessary for preventing the formation of thrombus in animals are below the haemorrhagic risk level. Consequently the retention of the antithrombotic properties of Heparin, reducing the haemorrhagic risk, are highly interesting. In this connection, various processes of degrading heparin with chemical reagents or enzymatic agents are described in the literature. These processes, commonly known as depolymerization, have the objective of obtaining fragments with a mean molecular weight lower than the heparin used as raw material and preserving its polysaccharidic structure. Amongst these processes, those based on nitrous acid can be quoted, where the consequent fragments are characterized by a terminal residue consisting of 2-5 anhydromanose.



R=H, $-SO_3H$

Others are based on depolymerization in a sulfuric acid medium with a subsequent resulfatation with a chlorosulfonic acid mixture (Nagasaka K. et al. Archiv Biochem, Biophys 150, 451 (1972). French Patent Appl. No. 2.538.404) or by peroxide or peracid action in presence of metallic catalysts (WO 86/06729) or by B-elimination in an alkaline medium of alkyl or allyl esters of heparin (EP-A 0 040 144) resulting in the obtention of oligomers with a mean molecular weight of 2000 to 9000 Daltons possessing an insaturated terminal group as follows



The yield of this process is low, considering the precipitation of the depolymerized product in an aqueous or organic medium and, in cases where the final product is lyophilized before elimination of the non-desired subproducts, the range of the molecular weight distribution is enlarged due to the inclusion of very low molecular weight fractions. A further process consisting of the enzymatic depolymerization with heparinase (EP-A 0064452; B. Biol. Chem. 257 7310 (1982) is effected with diluted solutions with low yields.

The process described in this invention has the following characteristics :

(a) The initial partition of the raw material by ultrafiltration (commercial heparin) with the object of not submitting the molecular weight heparin fraction lower than 3000 Daltons to depolymerization in order to avoid the presence of fragments of 10-16 saccharides or less in the final product. Despite the demonstrated necessity of the existence of a specific pentasaccharidic sequence which binds the antithrombine III (ATIII), Thunberg L. et al Carbohydr. Res. 100, 393-410 (1982), Petitou, M. Nouv. Rev. Fr. Hematol 26, 221-231 (1984), the decasaccharide or pentasaccharide fragments inhibit the Xa factor in the presence of V/Ca^{++}/Phospholipid Complex less than the eikosaccharides. Barrowcliffe, T.W. et al. Biochem J. 243, 31-37 (1987).

(b) Continuous operation in liquid phases without intermediate insulation, reduces the loss associated with precipitation and filtration.

(c) Elimination, prior to depolymerization, by phase partition, of low anionicity heparin fractions which, if included, would provide low or no specificity for the antithrombine (ATIII). Hurst R.E. et al. Biochim. Biophys Acta, 497, 539-547 (1977) have studied the partition of polyanionic glycosaminoglycan complexes with quaternary ammonium cations between alcoholic liquid phases and inorganic salt aqueous solutions. In the present invention, partition is performed between an aqueous saline solution and dichlormetane solution, the latter solvent being used for carrying the heparin in its esterifying, depolymerizing and molecular weight partition steps.

(d) Depolymerization effected in various cycles with the object of obtaining with a high yield a product with a defined molecular weight range lower than 5000 Daltons and a previously selected mean molecular weight.

As indicated, the B-elimination, by the action of a base on the esterified carboxyl of heparin is one of the depolymerizating chemical methods used. Up to the present, this has been used in the aqueous or organic phase, isolating the ester and consequently acting the base on the ester in one stage only. In order to obtain the mean molecular weight desired, other authors have studied the esterification grade control, the elimination time and the base's concentration. Before this invention, the results were a dispersion of molecular weights above 8000 Daltons which, in cases of mean molecular weights of 3000-5000 Daltons, represents a high contribution of extreme molecular weight fractions in the final product or the inclusion of a further fractionation stage subsequent to the depolymerization with the consequent reduction in yields.

The process, subject of the present invention is applied to aqueous solutions of commercial heparin at a 1 to 10% concentration (more specifically 5%). The first stage consists in the ultrafiltration of this solution through a hollow fiber cartridge H10 P3-2025 AMICON of 3000 NMWL in order to eliminate molecular weight fractions lower than 3000 Daltons. Fig.1a shows the molecular weight distribution curve of the initial heparin, Fig.1b the rejection of 3000 and Fig.1c permeation.

Although heparin is insoluble in organic solvents, its complexes with quaternary ammonium cations such as arylalkylammoniums are, under certain conditions, soluble in dichloromethane. These complexes have been proved susceptible of partition between aqueous solution of an electrolyte and dichloromethane, being the partition's coefficient a function of the electrolyte used and it's concentration. Partition is sensitive to the anionic nature of the glycosaminoglycan and, therefore, allows an adequate segregation of the low anionicity heparin fraction. Consequently, the rejection of the ultrafiltration with the hollow fiber cartridge, consisting of an aqueous solution of heparin is submitted to the addition of sodium chloride up to a concentration of 0.17M and a volume equal to 5% of the solution treated, of a bentethonium chloride solution at 7,5% in dichloromethane, and equilibrated with a 0.17M solution of sodium chloride in water.

Most of the heparin passes to the dichloromethane (SI) with the formation of a heparinquaternary ammonium complex and the separation of a low anionicity fraction in the saline aqueous phase (SII) (lower sulfate : uronic acid ratio) is verified.

Once the (SI) dichloromethane phase containing the heparin-quaternary ammonium is separated, the bentylic ester of the heparin's carboxylic acid is obtained by adding benzyl chloride to the (SI) solution (4-8 hours, 20-25° C).

The organic base, bentyltrimethylammonium hydroxide is subsequently added without isolation of the ester on the benzylated complex at room temperature (20-25° C) and the reaction proceeds for 2-4 hours. The depolymerization reaction is interrupted by the addition of 1N hydrochloric acid.

The depolymerized heparin fraction is separated from the nondepolymerized by phase partition between the solution in dichloromethane and an aqueous solution of 0.07M - 0.12 N sodium chloride, previously equilibrated with dichloromethane.

The depolymerized benzyl heparinate contained in the aqueous/saline solution (SIII) is debenzylized by treatment with 1N to 2N sodium hydroxide at a low temperature (0-5° C) for 1 to 4 hours.

The dichloromethane solution (SIV) containing the benzyl heparinate complexed with quaternary ammonium is once again treated with the organic base (HB).

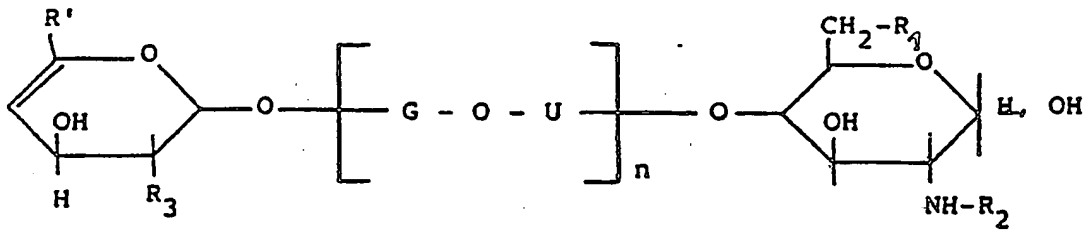
Rupture of the quaternary ammonium complex is effected by partition of phases between the dichloromethane solution and a 1.2M sodium chloride solution. The passage of all the depolymerized benzylated heparin to the saline aqueous phase (SV) is verified.

The saline aqueous solutions of the depolymerized heparin from the debenzylation step are dialysed by ultrafiltration in 1000NMW membranes and lyophilized.

The oligosaccharides resulting from the present depolymerization process can also be isolated from the aqueous solution of the dialysis by precipitation with nonaqueous solvents such as methanol, ethanol or acetone essentially in their sodium salt form.

The oligosaccharides of the present invention can also be salified with lithium, potassium, calcium or magnesium by the usual process, consisting in releasing the corresponding heparinic acid by means of an interchangeable cationic resine in proton form and subsequent salification of the heparinic acid with the desired cation.

The oligosaccharides of this invention respond to the following formula:



The double bond between C4 and C5 resulting from the β-elimination may or may not exist, depending on whether the product after debenzylation and dialysis, is treated with alkaline permanganate or sodium borohydride.

R' represents a hydrogen atom or a carboxyl group in free or salified form. R1 respresents an oxhydryl or sulfate group in acid or salified form. R2 designs a sulfonated group in acid or salified form or an acetyl group. R3 designs an oxhydryl or sulfate group in acid or salified form. -O- designs an oxygen bridge. G represents glucosamine chains and U uronic acid chains (glucuronic acid, L-iduronic acid and sulfated L-iduronic acid) according to their presence in the original heparin's polysaccharidic structure.

The oligosaccharides of this invention have the following characteristics as sodium salts:

Uronic Acid content :	25 - 40%
Sulfur content :	10 - 13%
Nitrogen content :	1.8 - 2.5%
Specific rotary power in aqueous solution at 20° C $(\alpha)_D^{20}$:	+ 28° + 55°
Mean molecular weight :	3000 - 7000 D.

According to the mean molecular weights and range of same obtained from application of this process, the final products can be classified as follows :

Mean molecular weight :	1500-3000 D.	3000-5000 D.	5000-7000 D.
Anticoagulant activity in vitro USP XXI :	10-50	50-70	70-120
AntiXa activity in vitro :	90-160	90-180	120-180
APTT activity in vitro :	10-40	30-60	60-110
$\frac{\text{AntiXa activity in vitro}}{\text{APTT activity in vitro}}$:	4-10	2-6	1.5-3

The following examples demonstrate the invention without limiting its scope.

Example I :

A commercial heparin originating from hog mucosa with the following features was used:

Anticoagulant activity USP XXI (Std. WHO IV) :	187 o.d.b.
Uronic acid content :	31.6 o.d.b
Sulfur content :	11.8 o.d.b.
Nitrogen content :	2.1 o.d.b.
Specific rotary power in aqueous solution at 20° C $(\alpha)_D^{20}$:	+ 47.4°
Mean molecular weight (see Fig.1a) :	17,000 Daltons

300 g of Sodium Heparin are dissolved in 12 litres of distilled water. The solution is concentrated to half its original volume by ultrafiltration through membrane AMICON E-10 P3-2025 and dialyzed versus two volumes of distilled water by the same equipment. 59.7 g of sodium chloride and 300 ml of a solution of benzethonium Chloride at 7.5% in dichloromethane is added to the rejection obtained from this last operation. The solution obtained is extracted with 6.5 l of a 7.5% benzethonium chloride solution in dichloromethane which has been previously equilibrated with an aqueous solution 0.17M in sodium c hloride. The phases are separated by extraction or centrifuging and the dichloromethane phase is transferred to a jacketed reactor provided with a stirrer and stirred gently with 300 ml of pure benzyl chloride at a temperature of 20 to 25° C. The stirring is maintained during 6 hours at the same temperature, after which 60 ml of benzyltrimethylammonium hydroxide are added and the stirring continued 3 further hours. Subsequently 10 ml of 1N hydrochloric acid are added and this solution is extracted with 6 l of a 0.1M sodium c hloride aqueous solution.

400 ml of 2M NaOH are added to the saline solution and stirred during 2 hours at 4° C, neutralized with 1N HCl and then dialyzed with an ultrafiltration unit equiped with a membrane of 1000 D. molecular weight cut-off.

The dichloromethane solution from the phase partition with the sodium chloride 0.1N solution is recycled in the reactor and 60 ml of benzyltrimetylammonium hydroxide are added and maintained in agitation during 3 hours at 20 to 25° C. 10 ml of 1N hydrochloric acid are then added and the solution is extracted with 6 l of an aqueous sodium chloride 2M solution.

400 ml of 2M sodium hydroxide are added to the aqueous saline solution containing the benzyl heparinate and the solution is maintained in agitation during 2 hours at 2° C, then neutralized with 1M hydrochloric acid and dialyzed with an ultrafiltration membrane of 1000 NMW cut-off, until the chlorides are eliminated. The rejected solution is lyophi lized (P2). P1 and P2 are blended and 254 g of product are obtained with the following characteristics:

Mean molecular weight :	4.700
Weight average molecular weight (MW) :	5.600
Number average molecular weight (MN) :	3.800
Anticoagulant activity (USP XXI) :	53 U/mg.
AntiXa activity :	163 U/mg.
APTT Activity (Teien A et al. Thromb. Res. 11, 107 (1947) :	44 U/mg.
Polydispersion Q = MN/MN :	1.47
(See Fig. 2)	

Example II

The raw heparin used was the same sodium heparin as in Ex.I.

300 g of sodium heparin are dissolved in 12 l of distilled water. The solution is concentrated to half it's original volume by ultrafiltration with an AMICON H-10 P3-2025 membrane and dialyzed versus two volumes of distilled water with the same equipment, obtaining a rejection of 6 l , to which 59.7 g of sodium chloride are added. The phases are separated by extraction or centrifuging and the dichloromethane (SI) transferred to a jacketed reactor with an agitator. 300 ml of pure benzyl chloride are added and stirred gently at a temperature of 20 to 25° C. Agitation is maintained during 6 hours at this temperature, after which 60 ml of benzyltrimethylammonium are added continuing agitation 4 further hours at the same temperature. 10 m l of 1N hydrochloric acid are then added and the solution is extracted with 6 l of a 0.07M sodium chloride aqueous solution. 400 ml of 2N NaOH are added to the saline aqueous solution and agitation is maintained during 2 hours at 4° C, after which the solution is neutralized with 1M HCl and dialyzed with an ultrafiltration membrane of 1000 NMW cut-off. The product is lyophilized (P1) and the dichloromethane solution from the partition phase with the 0.7M sodium chloride is recycled, 60 ml of benzyl chloride added and gentle agitation maintained during 6 hours at 20 to 25° C. 20 ml of 1M hydrochloric acid are then added and the solution is extracted with 6 l of a 2M sodium chloride aqueous solution.

To the saline aqueous solution containing the benzyl heparinate, 100 ml of 2N sodium hydroxide are added and agitation continued during 2 further hours at 4° C, after which the solution is neutralized with 1N hydrochloric acid and dialyzed with an ultrafiltration membrane of 1000 NMW cut-off until the chlorides are eliminated.

The rejected solution is lyophilized (P2) and blended with P1, obtaining 228 g of product with the following characteristics :

Mean molecular weight :	2900
Average molecular weight (MW) :	3600
Number average molecular weight (MW) :	2300
Polydispersion Q = MW/MN :	1.56
Anticoagulant activity (USP XXI) :	30 U/mg
AntiXa activity :	154 U/mg
APTT activity :	19 U/mg.
(See Fig. 3)	

Example III

A commercial heparin originating from bovine lungs was used as raw material with the following characteristics:

Anticoagulant activity USP XXI :	165 U/mg.
Uronic acid content :	33.2% o.d.b.
Sulfur content :	12% o.d.b.
Nitrogen content :	2.4% o.d.b.
Rotary power in aqueous solution $(\alpha)_D^{20}$:	+ 44,7°
Mean molecular weight :	16,000 Daltons

300 g of sodium heparin are dissolved in 12 l of distilled water. The solution is concentrated to half its original volume by ultrafiltration with an AMICON H-10 P3-2025 membrane and dialyzed versus two volumes of distilled water by the same equipment. 59.7 gr of sodium chloride and 300 ml of a 7.5% solution of benzethonium chloride are added to the rejection of the previous operation. The solution (S1) is extracted with a volume of 6.5 of a 7.5% solution of benzethonium chloride in dichloromethane previously equilibrated with a 0.17M sodium chloride aqueous solution. The phases are separated by extraction or centrifuging. The dichloromethane is transferred to a jacketed reactor with an agitator and 300 ml of pure benzyl chloride is gently mixed in at a temperature of 20 to 25°, maintaining agitation during 6 hours. After this operation, 10 ml of 1N hydrochloric acid are added and the solution is extracted with 6 l of a 0.12N sodium chloride aqueous solution.

400 ml of 2N NaOH are added to the saline aqueous solution which is maintained in agitation during 2 hours at 4° C and then neutralized with 1N HCl, dialyzed with an ultrafiltration membrane of 1000 NMW cut-off and lyophilized (P1).

100 ml of 2N sodium hydroxide are added to the aqueous saline solution containing the benzyl heparinate and maintained in gentle agitation for 2 hours at 4° C. The solution is then neutralized with 1N hydrochloric acid and dialyzed with an ultrafiltration membrane of 1000 NMW cut-off until the chlorides are eliminated. The rejected solution is lyophilized (P2). P1 and P2 are blended obtaining 273 g of product with the following characteristics:

Mean molecular weight :	5600
Average molecular weight (MW) :	6450
Number average molecular weight (NW) :	4100
Polydispersion Q = MW/MN :	1.57
Anticoagulant activity (USP XXI) :	73 U/mg
AntiXa activity :	171 U/mg
APTT activity :	64 U/mg

- The oligosaccharide mixtures obtained by the process described in this invention have been chemically and biologically characterised.

DISTRIBUTION OF MOLECULAR WEIGHTS

The molecular weight distribution was determinated by high pressure liquid chromatography in TSK 2000 and TSK 3000 colums using NaCl as the mobile phase. The elution profile was detected with the use of a UV (205 nm) detector.

Fig. 5 demonstrates the comparative distributions of the products obtained by : a) the process of this invention; b) depolymerization with nitrous acid; and c) β-elimination without fractionation.

In the first case, polydispersion is lower and even the mean molecular weights of (b) and (c) are apparently close (4800 and 5500), the contribution of high molecular weights is larger in (b) and (c) than in (a).

Consequently in (a), the contribution of molecular weight fractions higher than 7500 D, which potentiate the interaction of Cofactor II with thrombine, has been minimized.

Affinity chromatography on Sepharose ATIII demonstrates that the compound obtained with the invention contains more than 27% of the composition that binds to the ATIII. The latter is the consequence of the selectivity of the initial treatment by phase partition, eliminating the fractions of low anionic density, characterized by the low ratio of the content in O-Sulfate, N-Sulfate to Carboxylic groups.

ASSAY OF THE COAGULATION PARAMETERS.

AntiXa, antilla, TT and APTT in "in-vitro" treatment with blood from rabbits and dogs of the unfractioned heparin and oligosaccharide fractions, subject of this invention.

INACTIVATION OF THE Xa FACTOR

Animal : New Zealand rabbit.
Sex : Male
Treatment : Std. WHO 1 Low Molecular Weight Heparin (in vitro).
Assay : Chromogenic method antiXa KABI Coatest.

ANTI Xa FACTOR ACTIVITY (Absorbances)						
Animal N°	Concentration of substance assayed (UaXa/ml)					
	0.0	0.2	0.4	0.6	0.8	1.0
348	0.981	0.947	0.908	0.864	0.825	0.794
296	0.934	0.928	0.905	0.884	0.863	0.842
342	1.033	0.962	0.890	0.838	0.783	0.723
350	1.106	1.047	0.993	0.960	0.890	0.872
349	1.047	0.979	0.975	0.936	0.837	0.817
MEDIA	1.020	0.973	0.934	0.896	0.840	0.810
D.E.	0.0656	0.0456	0.0464	0.0506	0.0403	0.0564
E.E.M.	0.0293	0.0204	0.0208	0.0226	0.0180	0.0252
DIRECT LINEAR REGRESSION						
A = 1.0184 B = -0.2124	r = -0.998-0					
Treatment : H-BPM-B (in vitro) (Oligosaccharide fraction (Example II)						
348	-	0.101	0.208	0.308	0.312	0.482
296	-	0.261	0.482	0.698	0.840	1.080
342	-	0.251	0.595	0.873	0.920	1.588
350	-	0.232	0.402	0.797	0.957	1.188
349	-	-0.012	0.232	0.355	0.557	0.760
MEDIA	-	0.167	0.398	0.606	0.717	1.020
D.E.	-	0.1189	0.1478	0.2589	0.2755	0.4217
E.E.M.	-	0.0532	0.0661	0.1158	0.1232	0.1886
DIRECT LINEAR REGRESSION						
A = -0.0259 B = 1.0125	r = 0.9920					

Animal : Beagle dog Sex : Male Treatment : H-BPM-B (in vitro) (Std. WHO I-LMWH)						
ANTI Xa FACTOR ACTIVITY (Absorbances)						
Animal N°	Concentration of substance assayed (UaXa/ml).					
	0.0	0.2	0.4	0.6	0.8	1.0
6HL6	1.143	0.977	0.780	0.684	0.567	0.489
6HQ3	1.202	1.063	0.822	0.714	0.597	0.493
6HQ4	1.171	0.986	0.780	0.646	0.541	0.450
6GK4	1.164	1.015	0.830	0.678	0.580	0.496
6HO4	1.219	0.963	0.818	0.642	0.542	0.439
MEDIA	1.180	1.001	0.806	0.673	0.565	0.473
D.E.	0.0305	0.0396	0.0241	0.0297	0.0243	0.0268
E.E.M.	0.0136	0.0177	0.0108	0.0133	0.0109	0.0120
DIRECT LINEAR REGRESSION						
A = 1.1384 B = -0.7109	r = -0.989-3					
Treatment : H-BPM-B (in vitro) (Oligosaccharide fraction Example II)						
6HL6	-	0.371	0.621	0.752	0.877	0.974
6HQ3	-	0.321	0.583	0.707	0.912	0.991
6HQ4	-	0.335	0.606	0.736	0.871	0.968
6GK4	-	0.362	0.568	0.729	0.880	0.960
6HO4	-	0.295	0.589	0.711	0.860	0.963
MEDIA	-	0.337	0.593	0.727	0.880	0.971
D. E.	-	0.0308	0.0206	0.0185	0.0195	0.0123
E.E.M.	-	0.0308	0.0092	0.0083	0.0087	0.0055
DIRECT LINEAR REGRESSION						
A = 0.2351 B = 0.7775	r = 0.9839					

The AntiXa Factor activities tested with dog and rabbit plasma are similar for the unfractioned heparin and the oligosaccharide fractions. Figs. 6 and 7.

INHIBITION OF IIa FACTOR

A chromogenic assay was performed with Coatest Kabi S.2238 Kit. Human plasma, poor in platelets and defibrinated was used for this assay. The following figure demonstrates the results obtained with unfractioned heparin (H) and an oligosaccharide fraction (HBPM-B), the subject of this invention.

At the same concentration in plasma, a lower inhibition of thrombine is demonstrated for HBPM-B. Fig. VIII.

THROMBINE TIME

The thrombine time, consisting of the time required for the plasma to generate thrombine has been determinated in plasma from rabbits and dogs. In both cases, substantial differences were observed between the unfractioned heparin and the oligosaccharides (HBPM-B). For comparison purposes, two commercial depolymerized heparins (HBPM-C and HBPM-S) were analysed simultaneously with the

oligosaccharides (HBPM-B). Figs. IX and X.

Animal : N.Z. rabbit Sex : Male						
Treatment	THROMBINE TIME (seconds) (MEAN VALUES)					
	Concentration of substance assayed (U/ml)					
	0.0	0.2	0.4	0.6	0.8	1.0
H	18.0	54.4	137.7	>250	>250	>250
H-BPM-B	18.0	17.2	19.1	19.9	20.7	22.1
H-BPM-S	18.0	17.5	19.3	20.9	23.2	25.3
H-BPM-C	18.0	19.1	22.4	23.6	26.4	31.0

STATISTIC ANALYSIS (2 FACTORS WITH INTERACTION)	
Between treatments, global level	p 0.001
H/H-BPM-B	p 0.001
H/H-BPM-S	p 0.001
H/H-BPM-C	p 0.001
H-BPM-B/H-BPM-S	NS
H-BPM-B/H-BPM-C	NS
H-BPM-S/H-BPM-C	NS

Animal :Beagle dog Sex : Male						
H	9.9	19.6	52.5	116.0	>250	>250
H-BPM-B	9.9	10.1	10.5	10.7	11.4	11.8
H-BPM-S	9.9	10.2	10.8	11.9	12.7	13.8
H-BPM-C	9.9	10.7	11.8	13.0	14.5	16.6

STATISTIC ANALYSIS (2 FACTORS WITH INTERACTION)	
Between treatments, global level	p 0.001
H/H-BPM-B	p 0.001
H/H-BPM-S	p 0.001
H/H-BPM-C	p 0.001
H-BPM-B/H-BPM-S	NS
H-BPM-B/H-BPM-C	NS
H-BPM-S/H-BPM-C	NS

The following Tables show the Thrombine Time for different concentrations of Thrombine added to the plasma with various concentrations of glycosaminoglycans in plasma.

THROMBINE TIME (sec)	
(thrombine = 6 U/ml)	
CONTROL	5.5
SUH (2 ug/ml)	39.6
SUH (1 ug/ml)	11.4
SUH (0.5 ug/ml)	7.1
Fraxiparine (42 ug/ml)	5.5
LMWH-B (2 ug/ml)	5.5
(thrombine = 4 U/ml)	
CONTROL	6.4
SUH (2 ug/ml)	59.5
Fraxiparine (2 ug/ml)	8.8
LMWH-B (2 ug/ml)	8.3
THROMBINE TIME (sec)	
(thrombine = 2 U/ml)	
CONTROL	11.2 ± 0.11
Fraxiparine (4 ug/ml	92.8 ± 9.8
N;WH-B (4 ug/ml	40.3 ± 4.2
Fraxiparine (w ug/ml)	33.4 ± 0.8
LMWH-B (2 ug/ml)	22.8 ± 1.0
Fraxiparine (1 ug/ml)	16.6 ± 0.1
LMWH-B (1 ug/ml)	14.9 ± 0.2
Fraxiparine (0.5 ug/ml)	12.8
LMWH-B (0.5 ug/ml)	12.8
SUH : Commercial heparin Fraxiparine® : Depoloymerized commercial heparin LMWH-B : Oligosaccharide obtained with the invention.	

There is no modification in the Thrombine Time at high concentrations of thrombine inplasma referred to the control for depolymerized heparins Nowever, the unfractioned heparin shows a considerable increase.

At lower concentrations of added Thrombine and increasing the concentration of LMWH-B, there is a favorable difference compared with Fraxiparine which indicates for LMWH-B a possible lower risk of haemorrhage.

ACTIVATED PARTIAL THROMBOPLASTINE TIME (APTT)

Boehringer Mannheim's evaluation kit was used with poor platelet plasma of dogs and rabbits. The subject (HBPMB) at various doses does not increase the APT time, contrary to the unfractioned heparin (H)-.Fig. XI,XII.

Animal : Beagle dog

Sex : Male

ACTIVATED PARTIAL THROMBOPLASTINE TIME (sec)

(Mean values)

Treatment	Concentration of substance assayed (U/ml)					
	0.0	0.2	0.4	0.6	0.8	1.0
H	15.3	18.6	25.3	32.2	40.2	59.5
H-BPM-B	15.3	15.3	16.1	18.0	17.6	18.4

STADISTICS ANALYSIS ANOVA 2 FACTORS WITH INTERACTION	
Between treatments at global level	p 0.001
H/H-BPM-B	p 0.001
H/H-BPM-S	p 0.001

Animal : New Zealand Rabbit Sex : Male						
H	47.4	78.4	154.1	200.0	232.5	>250
H-BPM-B	47.4	57.9	57.1	57.7	59.9	67.4

STADISTICS ANALYSIS ANOVA 2 FACTORA WITH INTERACTION	
Between treatments a global level	p 0.001
H/H-BPM-B	p 0.001

INFLUENCE ON THE PLATELET AGGREGATION.

Numerous studies suggest that the hemorrhagic effect of heparin is caused by its action on the platelet functions. Salzman et al (J. Clin. Invest., 65, 64, 1980) studied the influence of molecular weight on the interaction of heparin and platelets. They concluded that high molecular weight fractions (20,000) presented a higher reactivity than the low molecular weight fractions.

In the present study the antiaggregant activity of commercial heparin (H) and the subject of this invention (HBPMB) on rabbit washed platelets suspended on Tyrode was investigated. The platelet aggregation was provoked with a solution of collagen (100 µg/ml) referred to a saline control. The study concluded that, for equal doses, the subject (HBPMB) presented a much lower effect on platelet aggregation than commercial heparin (H), and thus lower hemorrhagic risk. Fig. XIII.

EVALUATION OF THE ANTITHROMBOTIC ACTIVITY IN RATS

The formation of thrombus was induced by ligature of the cava vein. Commercial heparin and the subject were administered in dosis of 200 UaXa/Kg and 50-100 and 200 UaXa/Kg respectively, by subcutaneous injection (s.c.) and both were referred to a control. The results show equal activity for both, the subject and commercial heparin, when administered in equal doses. However, the subject presents a much lower anti-F IIa activity, in comparison to commercial heparin, resulting in a much lower risk of haemorrhage with the same antithrombotic activity.

INFLUENCE ON BLEEDING TIME.

The influence on bleeding time was evaluated by the method of total tail transection. As shown on the table below, the results indicate a very significant reduction of bleeding time for the subject in comparison to commercial heparin.

TREATMENT	Dosis (UaXa/mg)	Bleeding Time (sec)
Control	-	309 ± 20
Com. Heparin	240	764 ± 40
Com. Heparin	100	659 ± 41
HBPMB	50	327 ± 20
HBPMB	100	361 ± 40
HBPMB	240	446 ± 30

INFLUENCE ON HUMAN PLATELET FACTOR 4 (PF4).

The PF4 is a physiological inhibitor of unfractioned heparin released by the platelets. The disappearance of human PF4 was studied in the rabbit after pre-treatment with standard unfractioned heparin (H) and the subject (HBPMB). Both were given i.v. and PF4 (60 µg/kg) was given by the same route. Immunoreactive PF4, in untreated rabbits, disappeared very rapidly, while in the presence of H and HBPMB this process was slower. As observed in the table below, the results indicate that HBPMB has in vivo a good affinity for PF4, although less than H on a ponderal basis.

Treatment	Dosis (mg/kg)	PF4 kinetic parameters	
		Co(ng/ml)	t1/2(min)
Saline	-	128.8	2.1
H	3.44	1055.4	13.9
H	0.86	776.2	7.2
HBPMB	0.86	457.6	3.4
HBPMB	3.44	831.7	5.6

STUDY OF TOXICITY

The toxicity of HBPMB was studied and the lethal dosis 50 (LD 50) was calculated. The subject of the study was given i.v. and s.c. When administered intravenously, the subject was given in one dosis at 10 ml/kg body weight, and the LD 50 was calculated to be 3,300 mg/kg. When a dosis of 4,000 mg/kg was given, HPBMB induced neurological symptoms (clonic convulsions), due to the toxicity of mucopolysaccharides. When HBPMB was administered subcutaneously the LD 50 was calculated to be greated than 6,700 mg/kg.

The described product, given its characteristics and properties, results appropriate for the prophylaxis and treatment of thromboembolic diseases Moreover, the subject has a higher rate of benefits/haemorrhagic risk in comparison to unfractioned heparin in haemodialysis.

The low incidence of HBPMB in the APTT and the Thrombine Time (TT), when given in the usual doses of 0,5 - 1.0 mg/kg. minimize biological control during application.

The product described results appropriate for the preparation of pyro-gen-free pharmaceuticals, injectable intravenously and subcutaneously. It is also possible to administer the product orally, e.g. in form of tric-resistant capsules.

As a consequence of the low molecular weight of the product, it can also liposome-encapsulation for its controlled release intravenously, or its topical administration.

When the product is compared to unfractioned heparin in studies of bioavailability, performed subcutaneously in rats, the former shows 93% values, while the later shows 48-60%.

The studies done "In vitro" with human skin in a Franz difussion cell demonstrate the passage of 100% of activity of the subject. Therefore, the product could be formulated adequately for topic ointments, creams, as well as for other transdermal delivery systems (e.g. occlusive patches, microreservoirs, etc.) Furthermore, the subject could also be formulated for suppositories and inhalators, and associated with venotonic agents or thrombolitics.

Claims

1. A process for the production of oligosaccharide fractions derived from heparin, provided of pharmacological properties such as high antithrombotic activity, low effect on platelet function, high bioavailability, very low haemorrhagic risk, and virtually no toxic effects.

2. A process according to claim 1, characterized by in that the starting aqueous heparin solution is, in the first step, ultrafiltered through membranes of 3,000 D nominal molecular weight cut-off, in order to exclude low molecular weight fractions of heparin from the depolymerization treatment.

3. A process according to Claims 1 and 2 characterized in that the rejected ultrafiltered heparin solution is phase partitioned as a quaternary ammonium complex formed with aqueous saline solution and dichloromethane, in order to segregate fractions of low anionicity i.e. low specifity for antithrombine, from the depolymerization treatment.

4. A process according to claim 1 characterize in that heparin solution prepared according to preceeding claims, is chemically depolymerized in more than one step, separating after each cycle, a defined depolymerized fraction from the reaction mixture by phase partition.

5. Oligosaccharide fractions derived from heparin obtained by the preceeding claims characterized by low polydispersion, a low percentage of fractions of molecular weight above 7,000 D, and a high specificity for AT III factor.

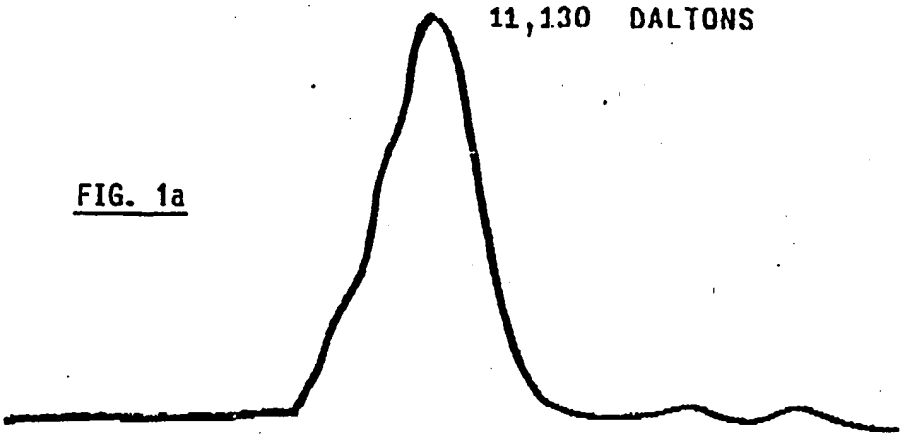


FIG. 1a

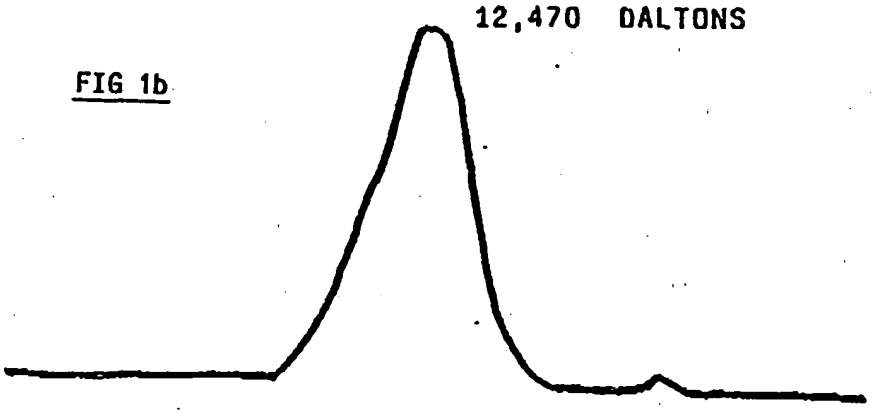


FIG 1b

FIG. 1c

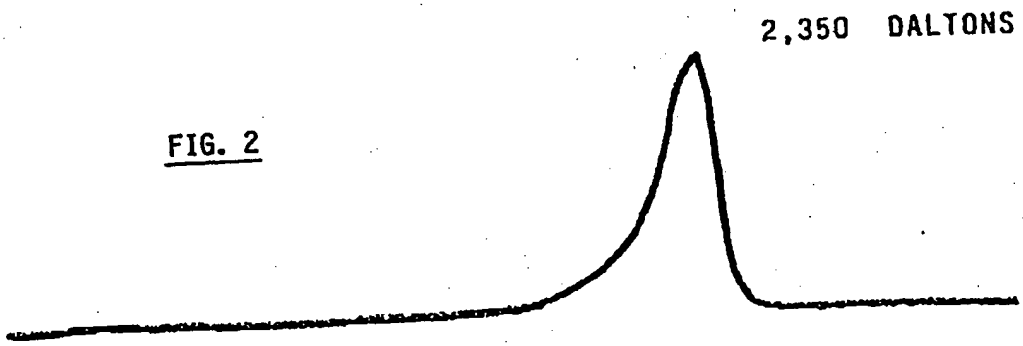


FIG. 2

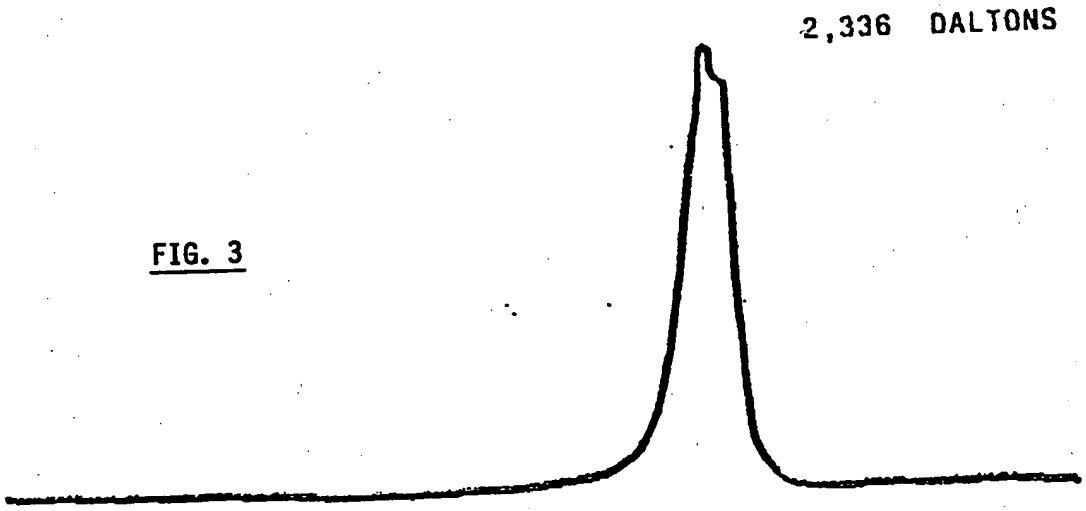


FIG. 3

FIG. 4

2,325 DALTONS

FIG. 5a

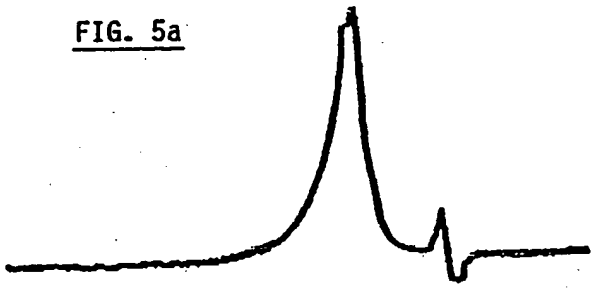


FIG. 5b

2,546 DALTONS

FIG. 5c

2,396 DALTONS

FIG. 6

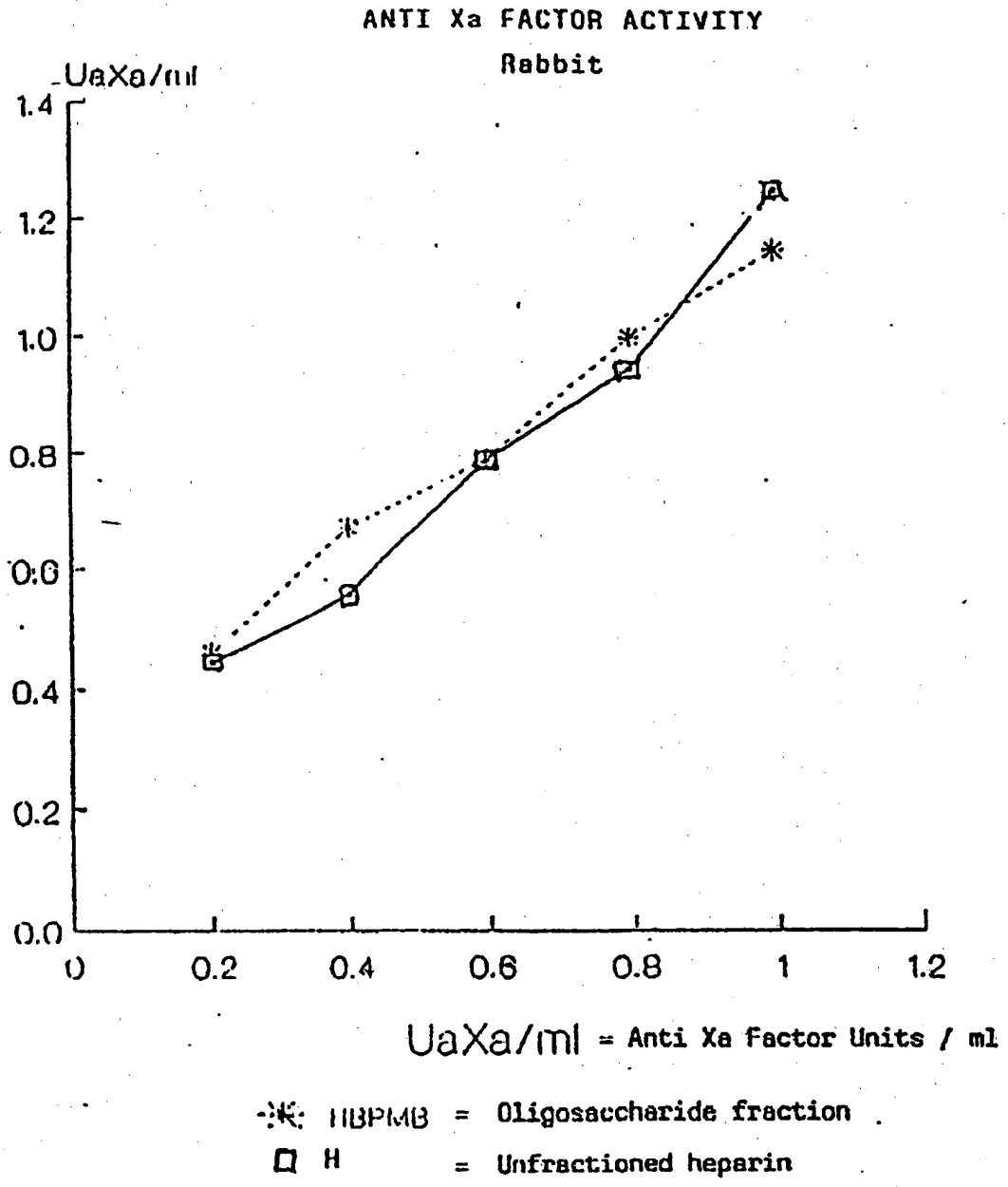

ANTI Xa FACTOR ACTIVITY
Rabbit
UaXa/ml
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.2
0.4
0.6
0.8
1
1.2
UaXa/ml = Anti Xa Factor Units / ml
HBPMB = Oligosaccharide fraction
H = Unfractioned heparin

FIG. 7

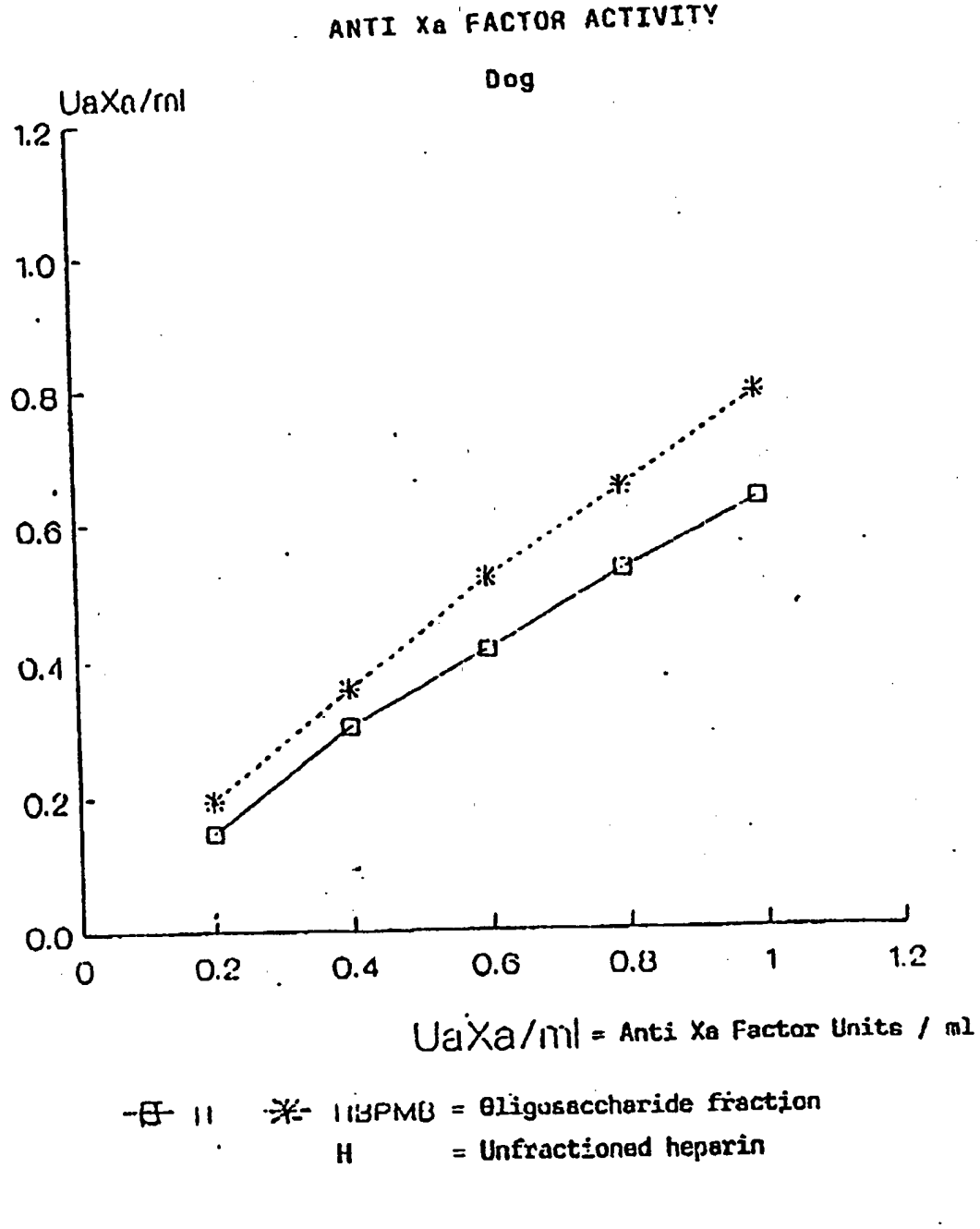
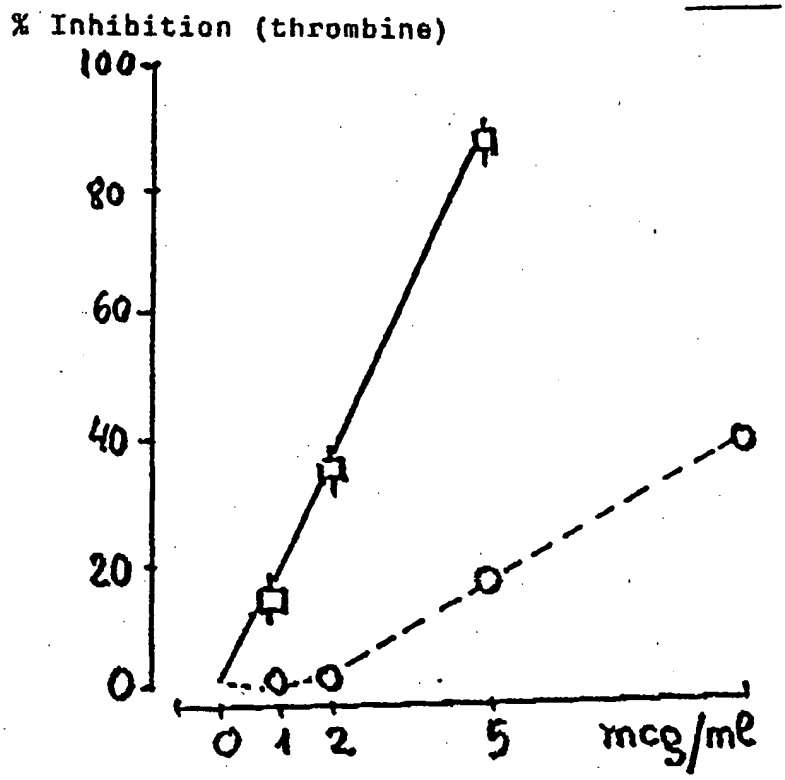

ANTI Xa FACTOR ACTIVITY
Dog
UaXa/ml
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.2
0.4
0.6
0.8
1
1.2
UaXa/ml = Anti Xa Factor Units / ml
HBPMB = Oligosaccharide fraction
H = Unfractioned heparin

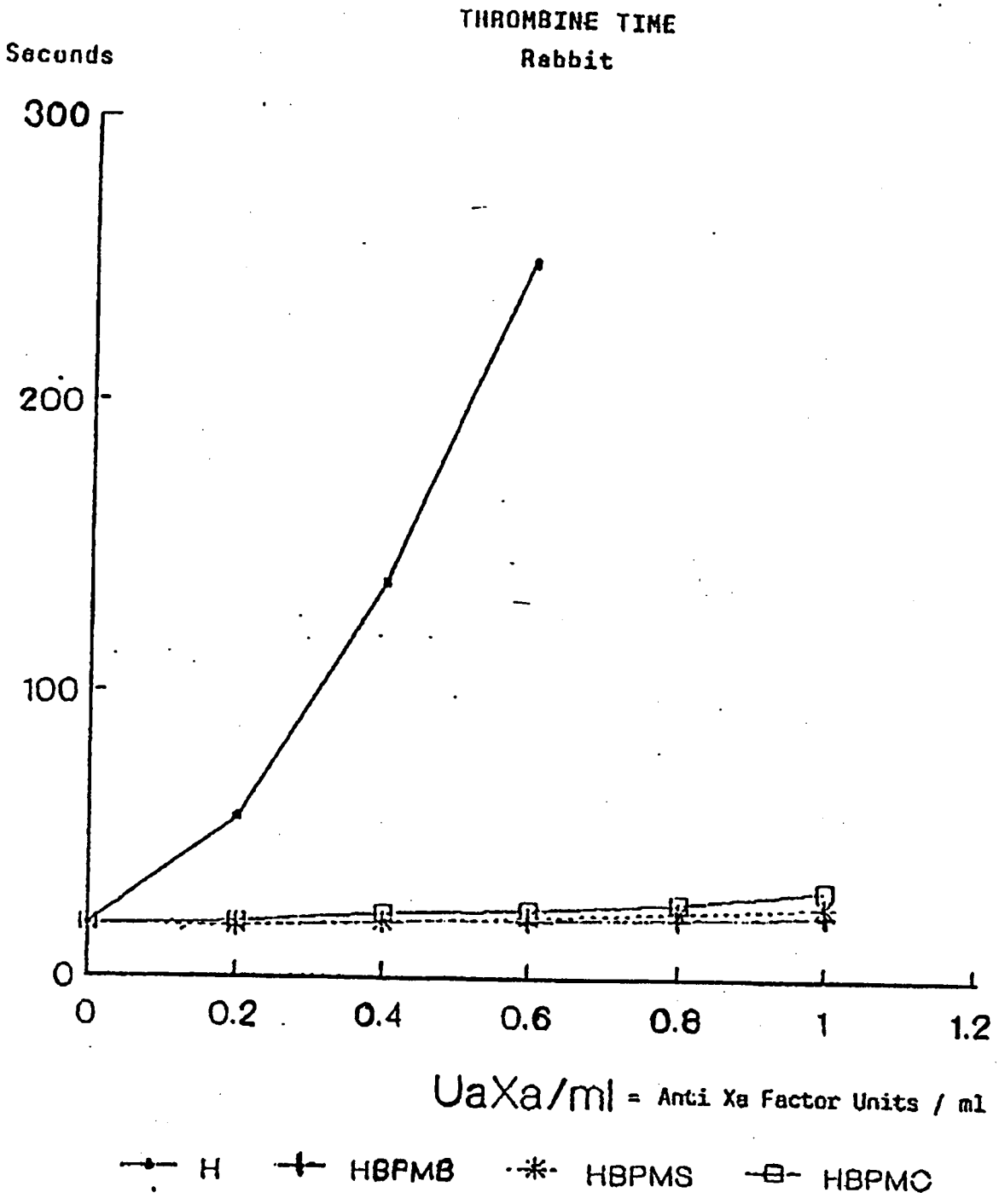
FIG. 8



□ H = Unfractioned heparin

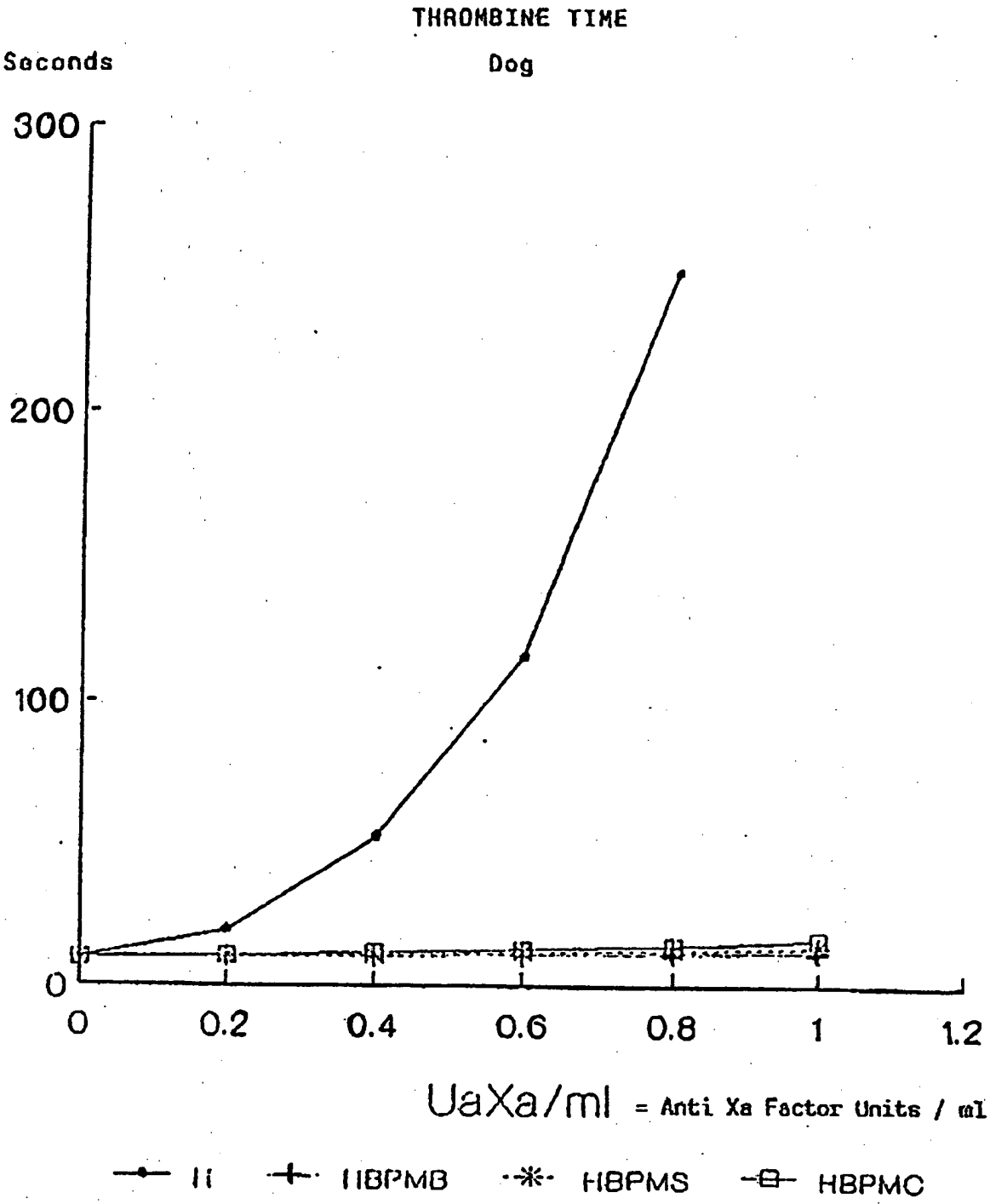
○ HBPMB = Oligosaccharide fraction

FIG 9



H = Unfractioned heparin
HBPMB = Oligosaccharide fraction
HBPMS = Commerciel depolymerized heparin
HBPMC = Commercial depolymerized heparin

FIG. 10



H = Unfractioned heparin
HBPMB = Oligosaccharide fraction
HBPMS = Commercial depolymerized heparin
HBPMC = Commercial depolymerized heparin

FIG. 11

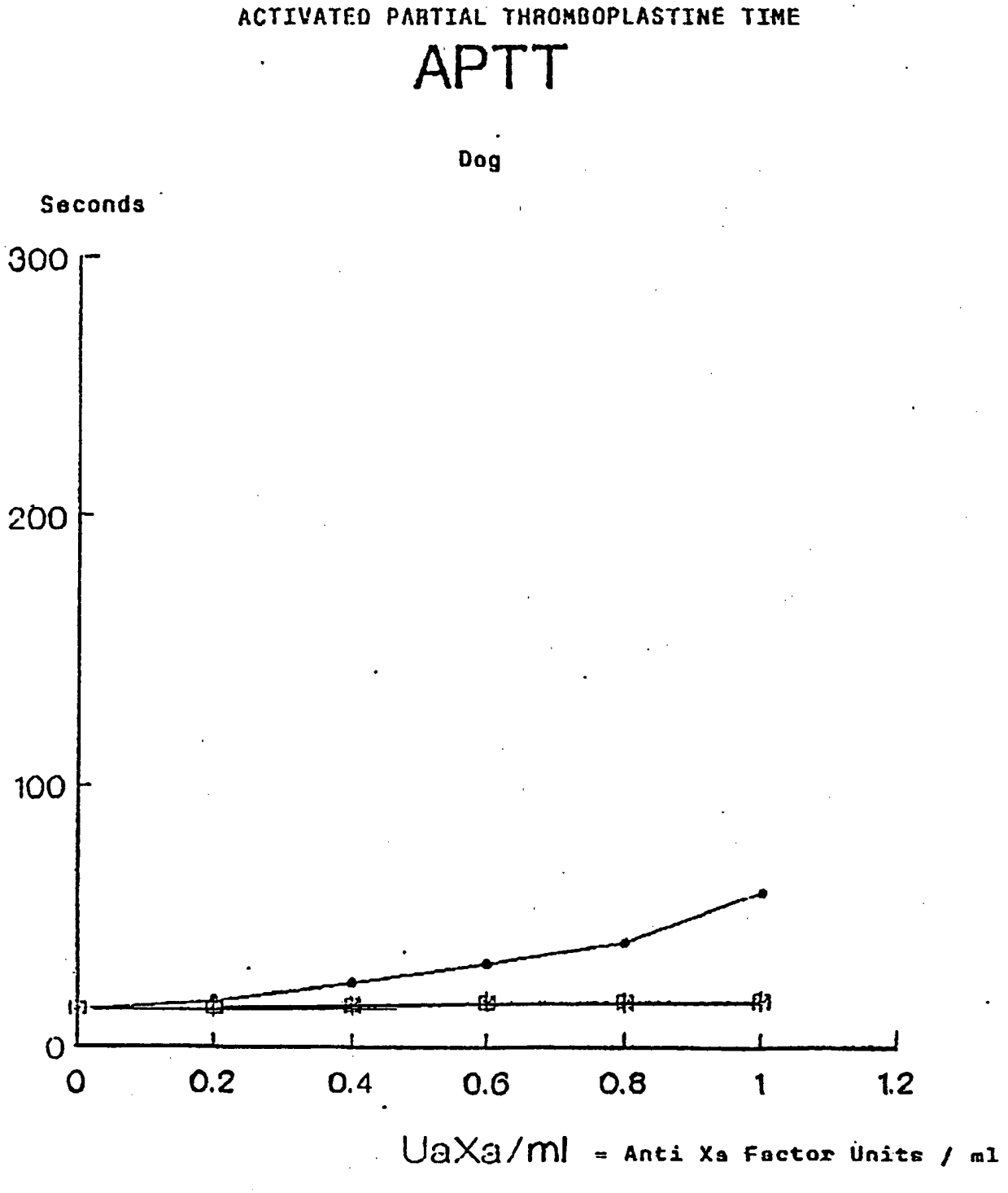
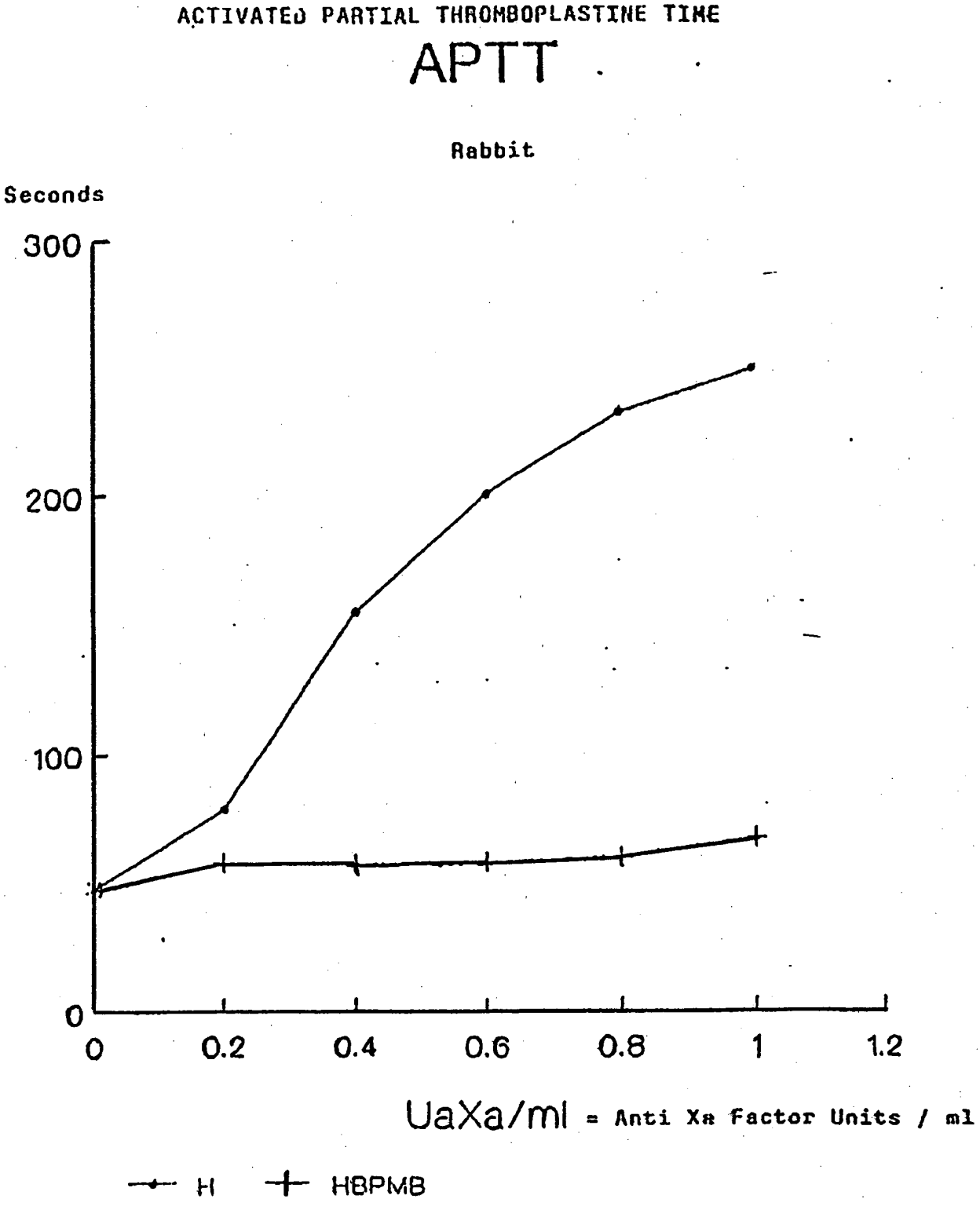

ACTIVATED PARTIAL THROMBOPLASTINE TIME
APTT
Dog
Seconds
300
200
100
0
0
0.2
0.4
0.6
0.8
1
1.2
UaXa/ml = Anti Xa Factor Units / ml
H
HBPMB
H = Unfractioned heparin
HBPMB = Oligosaccharide fraction

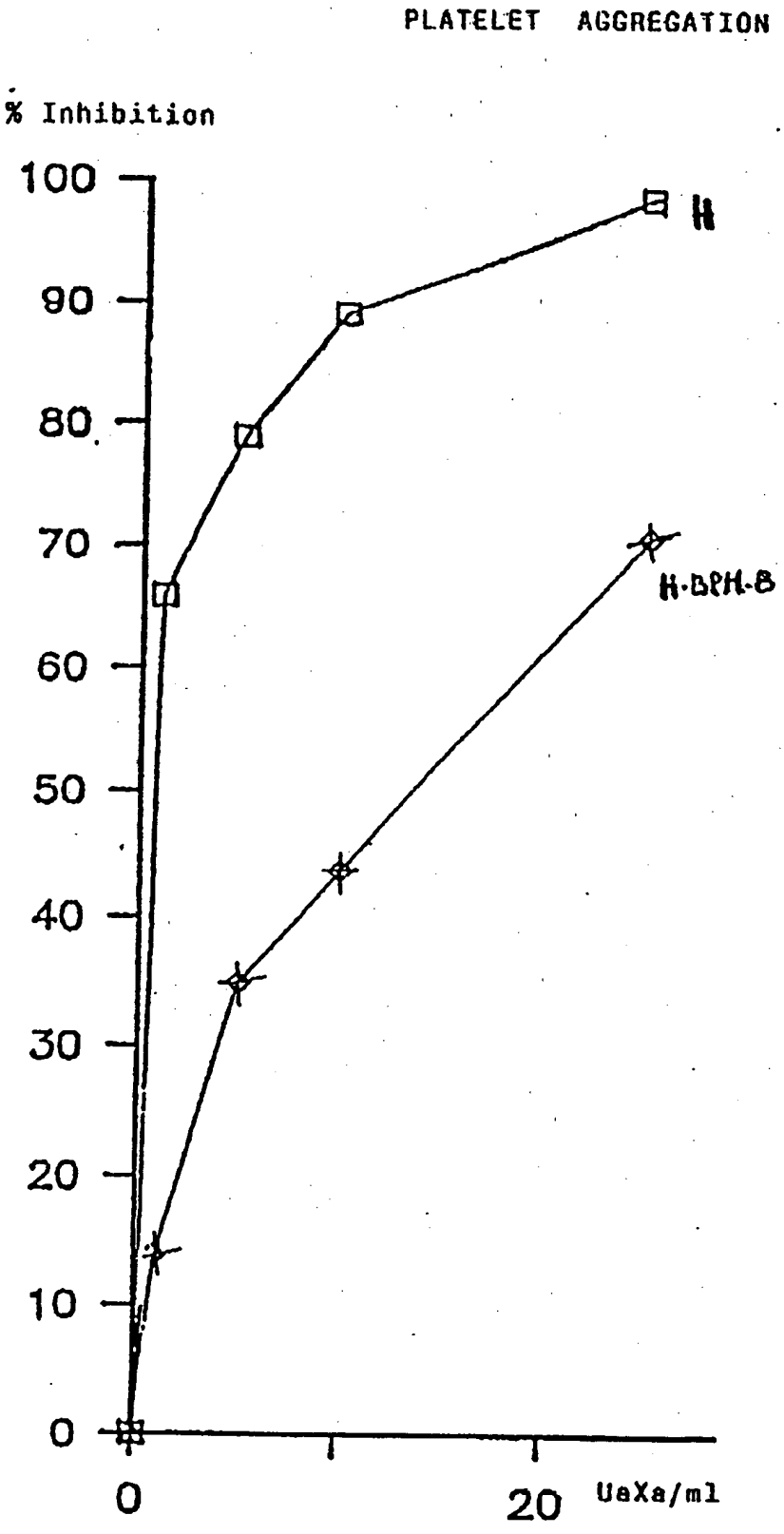
FIG. 12



H = Unfractioned heparin

HBPMB = Oligosaccharide fraction

FIG. 13



UaXa/ml = Anti Xa Fector Units / ml

H = Unfractioned heparin

H-BPM-B = Oligosaccharide fraction

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 10 6213

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,X	EP-A-0 040 144 (PHARMINDUSTRIE) * Claims 1,5; pages 7,8 *	1,5	C 08 B 37/10 A 61 K 31/725
X	US-A-4 533 549 (S.E. LASKER) * Abstract *	1	
A	WO-A-8 103 276 (RIKER LABORATORIES) * Claim 1; example 1 *	1,5	
A	BIOCHEMISTRY, vol. 18, no. 20, 2nd October 1979, pages 4283-4287, American Chemical Society, Washington, D.C., US; R.E. HURST et al.: "Structural basis for the anticoagulant activity of heparin. 1. Relationship to the number of charged groups"	1	
A	DE-A-2 945 595 (SANDOZ)		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 08 B
A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27-06-1989	SOMERVILLE F.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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